Kdr (Knock-down resistance) Detection to Cypermethrin in *Nezara viridula* (Homoptera:Pentatomidae)

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Abstract

A resistant strain of *Nezara viridula* selected by Cypermethrin was used to investigate the occurrence of knockdown resistance (kdr).Pretreatment of piperonyl butoxide (PBO) decreased RR from 20 to 7.5 folds to Cypermethrin. Electrophysiological studies showed that kdr ratio was 12.95 folds at EC50of burst discharge of neuromuscle preparation ,and was 16.32 folds at EC50 of blocked neuromusculartransmission.I've proved the point mutation in the para-homologus sodium channel gene associated with kdr upon genomic DNA. The kdr mutation ,G to C at nt 2999 resulting in a leucine to phenylalanine amino acid substitution ,was detected.

Introduction

Voltage-gated sodium channels are essential for action potential initiation and propagation in the nervous system and other excitable cells. In the past two decades, ten different mammalian sodium channel α-subunit genes were identified. It appears that the structural, functional and pharmacological diversity of mammal sodium channels is achieved primarily through expression of distinct sodium channel genes. In *Drosophila melanogaster*, however, para is the only gene that has been demonstrated to encode functional sodium channels.(Tan et al.,2004).

Voltage-gated sodium channels are targets of various neurotoxins including pyrethroid insecticides. Pyrethroid insecticides are widely used to control many agriculturally and medically important insect pests. Due to intensive use of pyrethroids, however, many pest populations have developed resistance to these compounds. One major mechanism of pyrethroid resistance, conferred by the knockdown resistance gene (*kdr*), is reduced target-site (sodium channel) sensitivity to DDT and pyrethroids. In the past several years we have

been characterizing the molecular mechanism of *kdr* in the German cockroach (Dong et al., 1998; Liu et al., 2002 and Tan et al., 2002),house fly(Williamson et al.1996) and *Heliothis virescens* (Park and Brown,2001). Results demonstrated that multiple point mutations in the sodium channel gene reduced the sodium channel sensitivity to pyrethroids, and that these mutations are likely responsible for *kdr* and *kdr*-type resistance to pyrethroids in insects. Demonstration of tight genetic linkage between knockdown resistance and the housefly gene encoding voltage-sensitive sodium channels spurred efforts to identify gene mutations associated with knockdown resistance and understand how these mutations confer a reduction in the sensitivity of the pyrethroid target site (Soderlund,2008).

This study aimed to detect knockdown resistance in the resistant strain of *N.viridula* to Cypermethrin upon electrophysiological studies and DNA analysis especially with almost none avilable papers in this way concerning *N.viridula* as an important pest able to transfer many plant viruses.

Materials and Methods

Insect

Colonies of ***N.viridula*** were reared as described by Corrêa-Ferreira (1999), under controlled conditions (25 ± 1 ºC and 65 ± 5% RH with a photoperiod of 16:08 (L: D).).

They were reared on bean plants with a small source of water, which were all in cages .All plants were cleaned before presented to insects. The beginning of these colonies was isolated from castor oil plants.

Assessment of Cypermethrin activity was conducted to evaluate the activity of tested pesticide against *N.viridula* adult females. The spraying technique described by Panizzi (2006) was used.

Rearing of *N.viridula* colonies under pesticides selection pressure

Treated insects colonies reared under selection pressure of Cypermethrin.It was carried out by spraying technique at LC50 level of the pesticide, from parents' generation till the 20th generation.

For studying the levels of resistance in these selected strains, toxicity lines of the inducer against the adult females were established every two generations. All the results of the selected strains were compared with those of the laboratory strain.

Chemicals

Cypermethrin(Pyrethroids group).Synergists used were technical grade of piperonyl butoxide(PBO)95%,and S,S,S-Tributyl phosphorotrithioate (DEF) 99% .

Bioassay

To screen for potential kdr individuals in resistant *N.viridula* adult females to Cypermethrin were first treated with a mixture of the oxidases inhibitor PBO (100 µl/insect) and the esterase inhibitor DEF (30 µl/insect) by topical application method. Droplets of 2-µl acetone solutions of Cypermethrin was applied to the dorsal region of each insect (30-40 mg) using a Hamilton micro syringe.Treatment of the insect with synergist alone did not produce mortality or knockdown.

In all bioassays, five doses plus a control were replicated at least three times with 10 insects per replicate. All treated insects were kept individually at 25±1ºC and L:D 16:8 and were supplied with clean bean plants with source of water.Knockdown was defined as the inability to exhibit coordinated walking after prodding with forceps(Dong et al.1998) .Data were analyzed by probit analysis. The lack of overlap between the 95% confidence limits of LD50 or KD50 was used as the criterion for significant difference ($P<0.05$).

Electrophysiological Assay for kdr

Electrophysiological experiments were performed according to Ru et al.(1998), to examine the neuronal sensitivity to Cypermethrin.. A preparation of the ventral longitudinal muscle cells of 20-30 mg *N.viridula* was used. The insect was pinned to a magnetic plastic-coated dish and dissected from the dorsal surface bathed in saline. The viscera, fat body, and other loose tissues were removed and the exposed body wall and associated nervous system were covered with saline. Spontaneous miniature excitatory junctional potentials (mEJP)were recorded with 3M kcl-filled glass microelectrodes of 15-25 MΩ resistance connected to a microelectrode amplifier and photographed from th screen of storage oscilloscope. mEJP was identified by amplitude discrimination. The frequency of mEJP over a 5-min control period in normal saline was recorded at first. After 5-min.intervals the preparation was immersed in fresh saline containing different concentrations (from low to high, respectively) of the insecticide.The pyrethroid was dissolved in acetone before dilution in the saline. Experiments were conducted at 15±1ºC.For each strain , 20 insects /concentration .Data were analyzed by probit analysis.Burst discharge EC50(BD-EC50)was defined as the concentrations of insecticides eliciting repetitive burst discharge responses from 50% of the individuals tested from each sample, and was calculated by probit analysis from plots of log

concentration versus probit of cumulative frequency. Transmission block EC50 (TB-EC50)was defined as the concentrations of insecticides eliciting neuromuscular transmission blockage responses from 50% of the individuals tested from each sample,and was calculated by probit analysis from plots of log concentration versus probit of cumulative frequency. The lack of overlap between the 95%CL of BD-EC50 or TB-EC50was used as the criterion for significant difference ($P<0.05$).

PCR and sequencing of *N.viridula*

DNA was extracted individually from the antennae, head or legs to avoid DNA contamination from parasitoids that could be present inside the body. The genomic DNA was isolated from *N.viridula* according to (Sosa-Gómez et al., 2004).

A sense primer skdr/Dg1-F(GCCCBAAGTACTATTTCCAGGARG) and an anti-sense primer kdr/2-R(GGAGAGATTGGAAGACCCAAAA) ,were used in PCR to amplify a 4.5-Kb fragment containing the IIS4-5 regions.Locations of the two primers in *N.viridula* are indicated in Fig.1.PCR procedure was continued upon Dong et al.(1998).Sequencing primers such as IR1 was used to linear amplify 220 and 230 bp.

Automated and manual ligand docking

The crystal structures of pyrethroid,Cypermethrin (Fig.3) was obtained from the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/). With the aid of AutoDock 3.0 software package, the voltage gated sodium channels were gained to make imagination of kdr so easy .

Resuts

Toxicity and Synergism

The toxicity of Cypermethrin alone and with synergists to (R) and (S) of *N.viridula* adult females are shown ion Table (1).The RR of R strain of Cypermethrin was 20 folds .The synergist and inhihibtor of esterases ,DEF,did not significantly synergize Cypermethrin in the resistant strain,its RRvalue was 25.3 folds (overlapping 95% CL).Pre-treatment with PBO decreased the resistance from 20 folds to 7.5 folds.Used synergists did not completely abolish the resistance ,it was then clearly that other mechanisms may also be involved.Slope values of dose –probit mortality curves were smaller for most resistant strains compared with those of susceptible strains,indicating heterogeneity of the resistant strains except in the case of synergism with PBO. Kdr evaluation as shown in Table (2), after treatment with PBO ,kdr was detected according to Dong et al.(1998).KD50 of resistant strain was 0.912(0.784-1.008),with RR 45.6 folds.Slope value

in case of R strain (3.68) was higher than that of S strain (4.52),suggesting nearly homogeneity of R strain.

Kdr Tested by Electrophysiological Assay

To measure the nerve insensitivity more directly,kdr was studied by an electrophysiological method according to Ru et al.(1998) .Table (2)showed that RR of R strain was 12.95 folds to Cypermethrin,at the EC50 of BD (Burst Discharge).While RR was16.32 folds to Cypermethrin,(Table 3),at the EC50 of TB (Transmission Block).

Sequence Analysis of Genomic DNA,Corresponding to the IIS3-IIS6 Region

To facilitate the detection of kdr or super kdr mutations in individual N.viridula by PCR,we chose genomic DNA isolated from individual N.viridula as the template in PCR because isolation of messenger RNA and subsequent synthesis of cDNA proved to be technically difficult and time consuming. Para genomic sequence (both exon and intron)were corresponding to the IIS4-IIS5 cDNA region. The mutations that cause resistance are most commonly found in the domain II region of the channel protein where five different residues have been implicated to date: Met918 in the IIS4-IIS5 linker, Leu925, Thr929 and Leu932 in IIS5 and Leu1014 in IIS6. The most common mutation is L1014F .The 4.6kb DNA fragment was gel-purified and sequenced by primer walking.The exon sequence in this part was interrupted by the intron of 2kb .The kdr mutation at nt2999 is located 3bp upstream of the 2kb intron.(Figs.1 and 2).Under the conditions used in this study ,the success rate of sequencing PCR-amplified DNA fragments was 100%.

The exons were identical to the correspondence cDNA sequences, including nt 2999 where the kdr mutations were found:
G*2999 and C*2999 in the Cypermethrin resistant strain .Beside G*2877, which improved possibilities of alternative exons and particular sequences that would indicate generation of G*2999/C*2999 by genomic or cDNA reorganization.

Depending on all previous results it can be concluded that the point mutation in the para – homologus sodium channel gene has shown to be linked to oxygenases mediated detoxication, beside IIS4-IIS5 cDNA regions and these findings are supported with PCR , which showed differences sequences of voltage gated sodium channels depending on oxidases .

Discussion

In this study, the occurrence of the kdr mutation was examined in Cypermethrin-resistant strain of *N.viridula* adult females. DEF did not synergize Cypermethrin in the resistant strain.It was suggested that esterase are not important for resistance to pyrethroids in R strain.After pretreatment with PBO,the resistance ratio decreased to the pesticide.

These results suggested that elevated mixed-function oxidases (MFO) mediated detoxification is an important factor in pyrethroids resistance.Our results are on the same trend with (Gunning et al.1991)showed that MFO mediated detoxification of pyrthroids is a primary mechanism in medium resistance level of *Helicoverpa armigera* (RR<50 Folds),however,kdr (nerve insensitivity) appeared to be the primary mechanism for higher resistance.Also , Valles and Yu 1998,supported our results by their work on *Blatella germanica* ,which pretreatment with PBO and DEF actually increased the cypermethrin resistance ratio by two folds .Concerning our results of the genomic organization of the point mutation of the para homologus sodium channel gene analysis were supported by Eleftherianos,et al.(2008), who metioned that recent advances in the characterisation of insect sodium channel gene sequences have identified a small number of point mutations within the channel protein that are implicated in conferring target-site resistance to pyrethroid insecticides (so-called knockdown resistance or kdr). The L1014F (leucine-to-phenylalanine) mutation located in the centre of segment 6 of the domain II region (IIS6) of the sodium channel (the so-called kdr trait) has been detected in the peach-potato aphid, *Myzus persicae* (Sulzer), and is considered to be the primary cause of pyrethroid resistance in this species. Beside that , a second mutation was characterized of, M918T (methione-to-threonine), within the nearby IIS4-S5 intracellular linker (the so-called super-kdr trait) in a field clone also possessing L1014F, with both mutations present in heterozygous form. The resistance phenotype of *M. persicae* clones possessing various combinations of L1014F and M918T to a wide range of pyrethroids (both Type I and II) was assessed in leaf-dip bioassays and to lambda-cyhalothrin applied at up to ten times the recommended field rate as foliar sprays to aphids feeding on whole plants. Bioassay results demonstrated that presence of both mutations was associated with extreme resistance to all the pyrethroids tested relative to aphids lacking the mutations. Furthermore, this resistance well exceeded that shown by aphids that were homozygous for L1014F but lacking M918T. Also, Martinez-Torres et al.(2009),who improved kdr mutation in Myzus persicae. The mutation was present in four clones with amplified E4 esterase genes, but was absent from both susceptible clones and those with amplified FE4 genes. The inferred presence of kdr-type resistance in the four E4 clones was subsequently confirmed by bioassays that showed this

to be the primary mechanism of resistance to deltamethrin and DDT, although the esterase-based mechanism also contributes to the overall level of deltamethrin resistance.

Automated docking of Cypermethrin with the model of the sodium channel was performed using the AutoDock 3.0 software package,used to help imagination of kdr resistance. In our model the α-cyano group on Cypermethrin is located between IIS5 and IIIS6 in the vicinity of Ile1533 (on IIIS6). For pyrethroids such as fenvalerate with an α-cyano substituent, there is potential for a hydrogen bonding network between the alpha carbon proton, the ester carbonyl group and the side-chain hydroxyl group of Thr929. It can be postulated that ,positioning of the α-proton is also key for the interaction of the 3-phenoxybenzyl alcohol pyrethroids, in that it provides an additional contact point between the pyrethroid and the sodium channel only in the presence of an aromatic ring substituent in the meta position. The activity of non-cyclic substituents or substituents at the para position is not enhanced by the presence of the α-cyano group. The hydrophobic face of the II S4-S5 linker forms the lowest section of a hydrophobic cavity that is also delimited by the S5 helix of domain II and the S6 helix of domain III. This cavity is lined by the side-chains of key residues previously implicated in pyrethroid binding, including Met^{918}, Leu^{925}, Thr^{929} and Leu^{932}. As the voltage sensor domains are not positioned to occlude the cavity (Figure 4 a,b), the site is predicted to be accessible to the lipid bilayer and thus lipid-soluble insecticide molecules. Docking studies of pyrethroids was therefore undertaken to assess whether this cavity could be the insecticide-binding site.

The sodium channel was modeled in an open conformation with the insecticide-binding site located in a hydrophobic cavity delimited by the domain II S4-S5 linker and the IIS5 and IIIS6 helices. The binding cavity is predicted to be accessible to the lipid bilayer and therefore to lipid-soluble insecticides. The binding of insecticides and the consequent formation of binding contacts across different channel elements could stabilize the channel when in an open state, which is consistent with the prolonged sodium tail currents induced by pyrethroids and DDT. In the closed state, the predicted alternative positioning of the domain II S4-S5 linker would result in disruption of pyrethroid-binding contacts, consistent with the observation that pyrethroids have their highest affinity for the open channel. The model also predicts a key role for the IIS5 and IIIS6 helices in insecticide binding. Some of the residues on the helices that form the putative binding contacts are not conserved between arthropod and non-arthropod species, which is consistent with their contribution to insecticide species selectivity. (O'Reilly et al.2006).

Phosphorylation of a single residue, serine 1506, that is located in the conserved intracellular loop between domains III and IV and is involved in inactivation of the sodium channel, is required for both modulatory effects. Mutant sodium channels lacking this

phosphorylation site have normal functional properties in unstimulated cells but do not respond to activation of protein kinase C. Phosphorylation of this conserved site in sodium channel alpha subunits(Fig.4c) may regulate electrical activity in a wide range of excitable cells (West,et al.2003). Residues Met918, Thr929, Leu925 and Leu932 are predicted to form close (≤4 Å) binding contacts with the pyrethroid, Cypermethrin. These interactions are in agreement with experimental data obtained from resistant insect populations where mutations at these positions in the sodium channel confer resistance to pyrethroids. Furthermore, the function of these mutations in conferring resistance has been confirmed by in vitro experiments using gene expression in *Xenopus oocytes*. (Lee and Soderlund,2001). In the case of the two super-kdr substitutions M918T and T929I, this resistance may be manifested through the elimination of polar interactions with the pyrethroid. As our model predicts that changing the alcohol moiety affects the activity of a pyrethroid (e.g. a second aromatic ring might provide a close fit within the cavity and thereby enhance pyrethroid affinity), it is also possible that the close fit suggested by the model would render the pyrethroid susceptible to displacement by mutations that produce small changes in the shape of the cavity, e.g. L925I.

Val410 and Leu1014 are two loci that confer kdr-type resistance to pyrethroids and DDT [10,44]. They are located near the centre of the IS6 and IIS6 helices respectively and are located within approx. 11 Å of each other in our predicted three-dimensional structure. However, both loci are positioned relatively distant to the predicted binding cavity. Mutations at these positions lower the affinity of the open channels for pyrethroids by 10–30-fold, and decrease the availability of open channel states due to enhanced closed-state inactivation, thereby limiting the number of high-affinity binding sites available for pyrethroids (Lee and Soderlund,2001). It is not clear how these mutations exert such an attenuating effect but their close proximity to the gating-hinge position on each helix suggests they could impede the bending motions of the S6 helices necessary for channel opening, consistent with the effect of L1014F and V410M in shifting the equilibrium towards the closed state of the channel. Such a mutation (T529F) in the middle of the S6 segment of the rat rKv1.4 channel has a profound influence on channel activation and deactivation, destabilizing the open state and accelerating the rate of channel deactivation (Zhou,1998). Another possibility is that mutation of Val410 or Leu1014 may sufficiently alter the packing of S5 and S6 helices to cause a displacement of residues on IIS5 and IIIS6 that are required for insecticide binding.(O'Reilly et al.2006).

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Table(1) Toxicity of Cypermethrin to (S)and (R) of *Nezara viridula* adult females.

Pesticide	(S)strain		(R)strain		
	LD50 (95%CL)	**slope**	**LD50 (95%CL)**	**slope**	**RR**
Cypermethrin	0.030(0.015-0.006)	2.78	0.598(0.481-0.662)	2.75	20
+PBO	0.012(0.008-0.017)	2.35	0.090(0.074-0.011)	2.99	7.5
+DEF	0.018(0.012-0.025)	3.01	0.455(0.325-0.584)	2.01	25.3

Table(2) Estimation of kdr of Cypermethrin to (S)and (R) of *Nezara viridula* adult females.

Strain	KD50 (95%CL)	Slope	RR
S	0.020(0.017-0.025)	3.68	1.0
R	0.912(0.784-1.008)	4.52	45.6

Table(3) Electrophysiological assay of Cypermethrin to (S) and (R) of *Nezara viridula* adult females.

Pesticide	Strain	Burst discharge EC50			Transmission Block EC50		
		BD-EC50	**Slope**	**RR**	**TB-EC50**	**Slope**	**RR**
Cypermethrin	**S**	5.27 4.17 - 6.77	1.89	1.0	45.24 40.84 - 51.3	1.09	1.0
	R	68.24 60.47 -76.22	1.54	12.95	738.33 700.36-502.07	1.59	16.32

ATTAATATAC **GCCCAAAGTACTATTT CCAGGARG** GAACGAGTTC CACTATTTGT TTGATCAGTT GGAATCACAG CATTATTATT ACTACTTTCA TTACCTGTAC TAGCAGGTGC AATTACAATA TTATTAACAG ATCGAAACTT TAATACATCA TTCTTTGACC CTTCAGGAGG GGGAGATCCC ATTCTTTATC AACACTTATT TTGATTCTTT GCTCACCCTG AACTTTACAT **GGAGAGATTGGAAGACCCAAAA** GATTAATTTC ACACATTATT AGTCAAGAAA GAGGAAAAAA CGAAACATTT GGAAATATCG GAATAATTTA TGCTATATTA GCTATTGGAA TTATAGGA

Figure (1) *Nezara viridula* Voltage gated sodium DNA(*NEZARA* control strain)

ATTAATATAC **GCCCAAAGTACTATTT CCAGGARG** GAACGAGTTC CACTATTTGT TTGATCAGTT GGAATCACAG CATTATTATT **T**CTACTTTCA TTACCTGTAC TAGCAGGTGC AATTACAATA **CAC** TTAACAG ATCGAAACTT TAATACATCA TTCTTTGACC CTTCAGGAGG GGGAGATCCC ATTCTTTATC AACACTT**C**TT TTGATTCTTT G**G**TCACCCTG AA**G**TTTACA**T** **GGAGAGATTGGAAGACCCAAAA** GATTAATTTC ACACATTATT AGTCAAGAAA GAGGAAAAAA CGAAACATTT **CCA**AATATCG GAATAATTTA **TCC**TATATTA **CCT**ATTGGAA T**TT**TAGG**G**

Figure (2) *Nezara viridula* Voltage gated sodium DNA(*NEZARA* Cypermethrin-resistant strain)

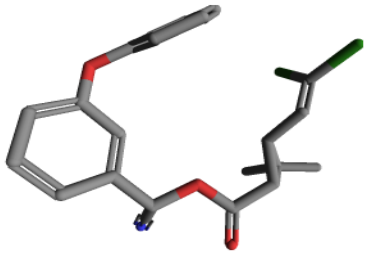

Figure (3) Cypermethrin structure by AUTODOCK program.

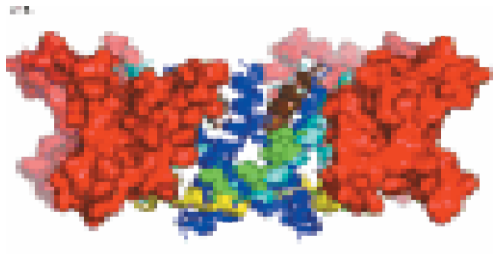

Figure (4.a)

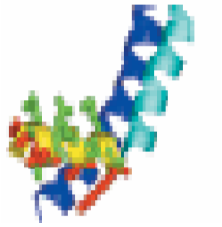

Figure (4.b)

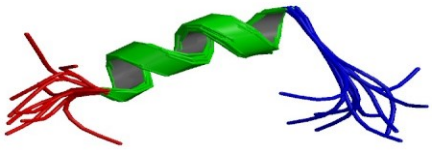

Figure (4.c)

Figure (4) Subunits helices of voltage gated sodium channel at activated state in *Nezara viridula*.

Figure (4.a.) Amphipathic α-helix.

Figure (4.b.) Opposite side of the α-helix is lined with hydrophobic residues, including Met918.

Figure (4.c) Voltage gated sodium channel, type VIII, α subunit.